

## BASIC RESEARCH STUDIES

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# Toward a mouse model of hind limb ischemia to test therapeutic angiogenesis

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**Introduction:** Several clinical trials are currently evaluating stem cell therapy for patients with critical limb ischemia that have no other surgical or endovascular options for revascularization. However, these trials are conducted with different protocols, including use of different stem cell populations and different injection protocols, providing little means to compare trials and guide therapy. Accordingly, we developed a murine model of severe ischemia to allow methodic testing of relevant clinical parameters.

**Methods:** High femoral artery ligation and total excision of the superficial femoral artery was performed on C57BL/6 mice. Mononuclear cells (MNCs) were isolated from the bone marrow of donor mice, characterized using fluorescence-activated cell sorting, and injected ( $5 \times 10^5$  to  $2 \times 10^6$ ) into the semimembranosus (proximal) or gastrocnemius (distal) muscle. Vascular and functional outcomes were measured using invasive Doppler imaging, laser Doppler perfusion imaging, and the Tarlov and ischemia scores. Histologic analysis included quantification of muscle fiber area and number as well as capillary density.

**Results:** Blood flow and functional outcomes were improved in MNC-treated mice compared with controls over 28 days (flow:  $P < .0001$ ; Tarlov:  $P = .0004$ ; ischemia score:  $P = .0002$ ). MNC-treated mice also showed greater gastrocnemius fiber area ( $P = .0053$ ) and increased capillary density ( $P = .0004$ ). Dose-response analysis showed increased angiogenesis and gastrocnemius fiber area but no changes in macroscopic vascular flow or functional scores. Overall functional outcomes in mice injected proximally to the ischemic area were similar to mice injected more distally, but muscle flow, capillary density, and gastrocnemius fiber area were increased ( $P < .05$ ).

**Conclusions:** High femoral ligation with complete excision of the superficial femoral artery is a reliable model of severe hind limb ischemia in C57BL/6 mice that shows a response to MNC treatment for functional and vascular outcomes. A dose response to the injection of MNCs appears to be present, at least microscopically, suggesting that an optimal cell number for stem cell therapy exists and that preclinical testing needs to be performed to optimally guide human trials. Injection of MNCs proximal to the site of ischemia may provide different outcomes compared with distal injection and warrants additional study. (J Vasc Surg 2012;56:1669-79.)

**Clinical Relevance:** Despite advances in surgical and endovascular technique, the options for many patients with critical limb ischemia remain limited. The injection of bone marrow-derived stem cells into ischemic tissue has been identified as a potential therapeutic alternative. Trial results have been consistently positive, yet the methods under which cell therapy is administered remain highly variable. The rationale for our study was to design a murine hind limb ischemia model capable of testing cell therapy parameters, such as optimal cell population, site of administration, and dose, and to use this preclinical knowledge to better guide and compare human trials.

The successful isolation of endothelial progenitor cells (EPCs) from the peripheral circulation in 1997 transformed the field of stem cell biology and created optimism for cell-based treatment of critical limb ischemia (CLI).<sup>1</sup>

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Although revascularization remains the current gold standard treatment of limb ischemia, many patients with advanced disease are not candidates for surgical or endovascular treatment secondary to anatomy and extent of their

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disease or comorbidities.<sup>2</sup> Despite advances in surgical and endovascular techniques, as well as advances in anesthesia and critical care, the options for these patients remain limited, with most ultimately requiring amputation.<sup>3-5</sup> At present, no standard effective treatment strategies are available for these no-option patients, and it is precisely for these patients that stem cell therapy holds the potential to create therapeutic alternatives. Accordingly, the use of bone marrow-derived stem cells has been identified as a potential method for inducing therapeutic angiogenesis.<sup>6</sup>

The publication of the Therapeutic Angiogenesis Using Cell Transplantation (TACT) trial in 2002 was the first report describing the use of bone marrow-derived mononuclear cells (MNCs) in humans for the treatment of CLI,<sup>7</sup> and several additional studies have been published since using MNCs derived from bone marrow<sup>8-16</sup> or peripheral blood.<sup>8,17-25</sup> Recent publications have been generally positive with regard to improvements in extremity perfusion and motor function.<sup>6</sup>

However, the greatest inconsistency in these reports comes from observed variability in MNC preparation, culture, and injection. MNC injections have been intramuscular,<sup>8-15,17,19-24</sup> and intra-arterial,<sup>9,18</sup> and between 80 and 1000 mL of bone marrow has been used.<sup>6,11,26</sup> In addition, the number of MNCs used per patient has varied widely, from  $0.1 \times 10^9$  to as high as  $100 \times 10^9$ . The number of injections given is equally variable, with only one study investigating the relationship between outcomes and number of injected cells.<sup>2,6,14,15,26</sup> Although the value of human studies cannot be underestimated, the differences between these studies, including variable degrees of ischemia, small patient numbers, and differing cell therapy techniques, prevent easy interpretation specifying the optimal techniques to use for common clinical practice.

Animal models are likely to play an important role in helping to answer some clinical questions to help guide practice and future trials of cell-based therapy for CLI. Several different murine hind limb ischemia models have been used to test angiogenesis via cell-based therapies, including models of mild and severe ischemia.<sup>27</sup> Variations in the level of occlusion include iliac ligation, femoral ligation below the branches, femoral ligation with excision of all branches, and artery and vein stripping, in addition to operator-dependent variations in technique, including suture ligation vs electrocautery.<sup>27,28</sup> Variation in the type of model used results in different patterns of ischemia and reperfusion, whereas the level of occlusion does not.<sup>29</sup>

Among the many uncertainties in therapeutic angiogenesis are the clinical questions regarding optimal cell population, site of administration, and cell dose. In this regard, the rationale for this study was to establish an acute but reproducible murine model of severe ischemia that would allow methodic testing of such parameters.

## METHODS

All procedures, protocols, and medications used in this study were approved by the Yale University Institutional Animal Care and Use Committee.

**Animal model.** Unilateral high femoral artery ligation and superficial femoral artery (SFA) excision was performed on 6- to 8-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Me). Intraperitoneal anesthesia was administered using ketamine (100 mg/kg) and xylazine (5 mg/kg). Mice were positioned in dorsal recumbency with their hind limbs externally rotated. A skin incision was made over the femoral artery beginning at the inguinal ligament and continued caudally to the popliteal bifurcation. The femoral artery was isolated above the level of the profunda and epigastric arterial branches, doubly ligated using 7-0 Prolene (Ethicon, Somerville, NJ) suture, and transected. The SFA caudal to the major branch points was dissected, ligated, and excised in its entirety.

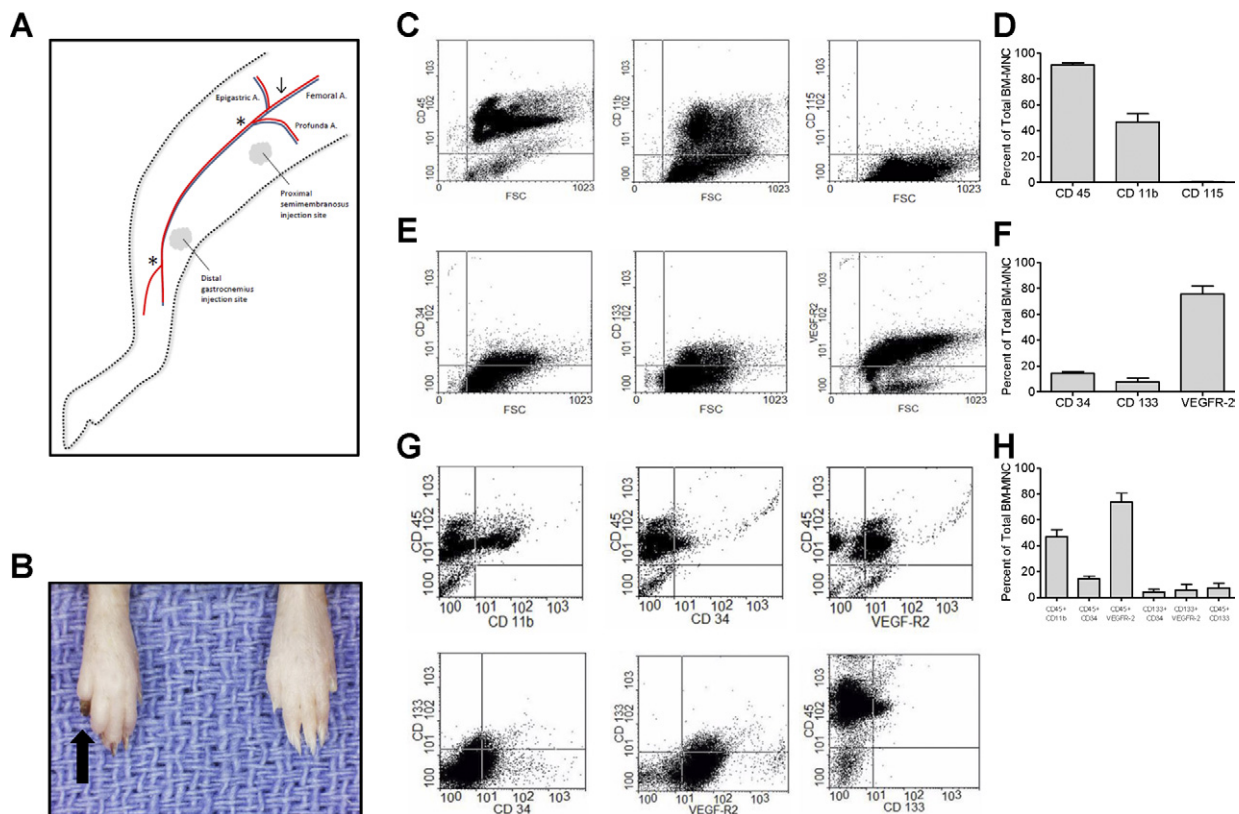
Varying concentrations of MNCs ( $5 \times 10^5$ ,  $1 \times 10^6$ , and  $2 \times 10^6$ ) or control medium, maintained at a constant volume of 0.1 mL, were injected distally into the gastrocnemius muscle (Figs 1-5) or proximally into the semimembranosus muscle (Fig 5) immediately after femoral artery ligation (Fig 1, A). A skin incision only was made on the contralateral limb for reference invasive monitoring. The incisions were closed, and body temperature was maintained with heating pads until the animals recovered from surgery and were ambulatory.

**Antibodies and reagents.** Primary antibodies to the following antigens were obtained as follows: cluster of differentiation (CD) 31 (Abcam, Cambridge, Mass); vascular endothelial growth factor receptor (VEGFR)-2-fluorescein isothiocyanate (FITC), CD11b-FITC, CD34-FITC, CD45-peridinin-chlorophyll protein, CD133-allophycocyanin, and CD115-phycoerythrin (eBioscience, San Diego, Calif); anti-fade mounting reagent with 4',6-diamidino-2-phenylindole, Alexa Fluor 568-conjugated immunoglobulin G, and Roswell Park Memorial Institute Media 1640 (Life Technologies, Carlsbad, Calif); and histopaque 1077 (Sigma-Aldrich, St. Louis, Mo).

**MNC isolation.** Bone marrow was collected from the long bones of euthanized C57BL/6 mice. Femurs and tibiae were harvested, clipped at the metaphyses, flushed with phosphate-buffered saline using a 26-gauge needle, and filtered through a 70- $\mu$ m cell strainer. MNCs were isolated by gradient centrifugation and resuspended in medium for immediate intramuscular injection. Cells isolated from one donor mouse at an injection concentration of  $5 \times 10^5$  could be used to treat ~4 to 6 mice.

**Fluorescence-activated cell sorting analysis.** MNCs were isolated from bone marrow as described and preserved with 0.5% paraformaldehyde. Aliquots were created, to which fluorescent-conjugated primary antibodies or isotype controls were added. Cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). WinMDI 2.9 software (Scripps Research Institute, La Jolla, Calif) was used to analyze data. Samples were normalized to immunoglobulin G isotype controls.

**Cutaneous perfusion measurement.** Laser Doppler perfusion imaging (LDPI; Perimed, North Royalton, Ohio) was used to measure perfusion of the ligated and



**Fig 1.** Methods. **A**, Modified hind limb ischemia model. The arrow ( $\downarrow$ ) denotes the site of femoral ligation, and the asterisks (\*) denote length of superficial femoral artery excision. **B**, Representative image of digit gangrene (black arrow). **C, D**, Expression of bone marrow mononuclear cell (BM-MNC) markers. **E, F**, Expression of endothelial progenitor cell markers. **G, H**, Coexpression of mononuclear cell and endothelial progenitor cell markers. **CD**, Cluster of differentiation; **FSC**, fluorophore-conjugated secondary antibody; **VEGFR**, vascular endothelial growth factor receptor. The error bars show the standard error of the mean.

control limbs at predetermined time points. Depilatory cream was used to remove hind limb hair, and the animals were kept on a heating pad at 37°C to minimize temperature variation. Consecutive measurements were obtained according to the manufacturer's protocols, and average hind limb perfusion was determined for an anatomically defined region of the lateral gastrocnemius and plantar foot by image analysis using LDPIwin 2.6 software (Perimed). Calculated perfusion was expressed as a ratio of right (ischemic) to left (control) hind limbs.

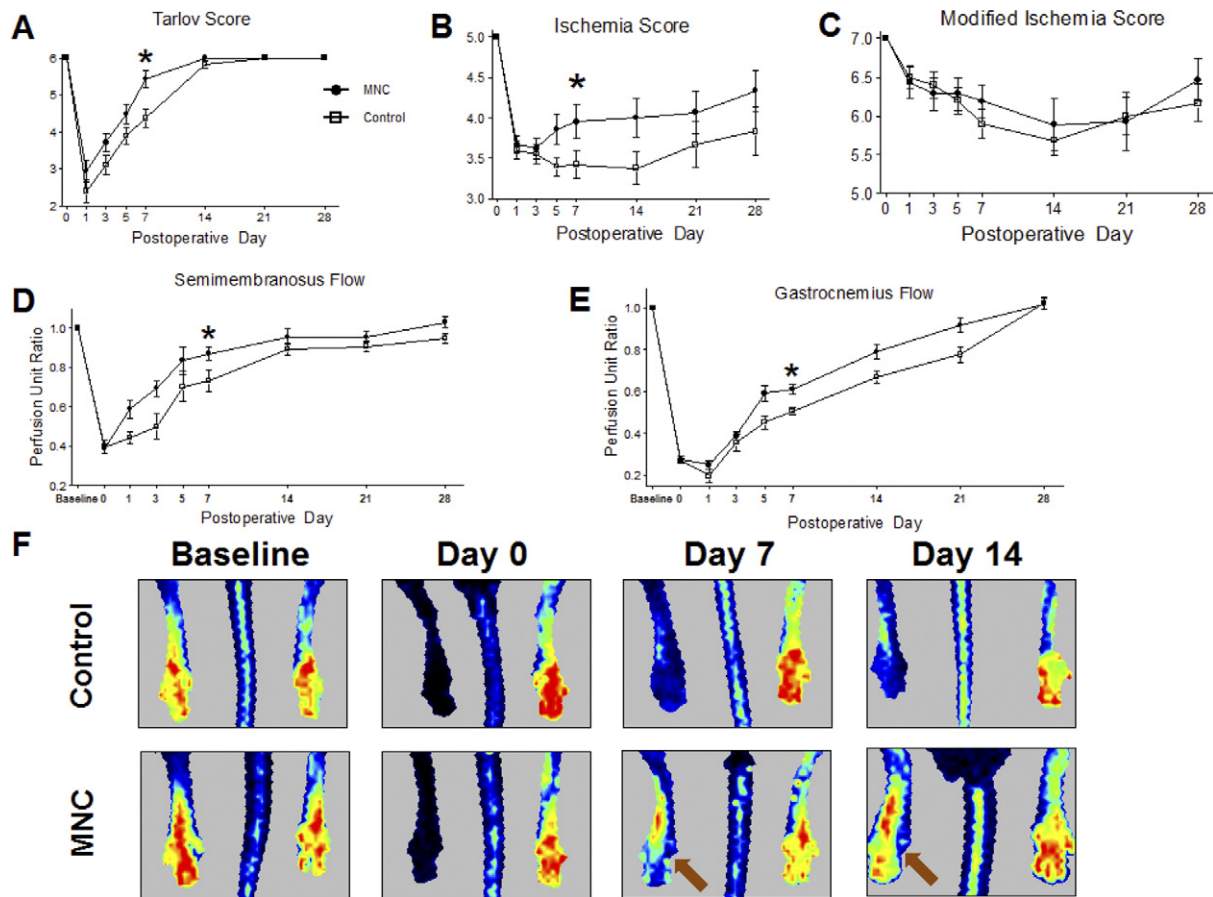
**Functional scoring.** Functional scoring was performed using the Tarlov scale,<sup>30,31</sup> ischemia scale,<sup>31,32</sup> and modified ischemia scale<sup>31</sup> (Table).

**Invasive Doppler flow measurement.** Blood flow was measured in some anesthetized animals in the ischemic leg and the control leg preoperatively, immediately postoperatively, and at 1 to 4 weeks after induction of hind limb ischemia, using the PeriFlux Laser Doppler Perfusion Measurement unit with a "deep probe" configuration (Perimed).<sup>31</sup> Access to the soleus muscle was obtained through a 3-mm skin incision on each hind limb that was closed after measurement. The same incision was used for all measure-

ments and was made only for mice having flow measurements and not for any other experiments. This method allows for reproducible measurements directly in the muscle bed, avoiding cutaneous blood flow and impaired wound healing, and is not significantly temperature-dependent. Blood flow values were expressed as the ratio of ischemic-to-control leg perfusion.<sup>31</sup>

**Histologic preparation and analysis.** At appropriate intervals after femoral artery ligation, animals were euthanized. Vessels were flushed with phosphate-buffered saline and heparin (250 U/kg). Perfusion-fixation was performed using 10% neutral buffered formalin solution, and the bilateral gastrocnemius muscles were removed. Tissue was embedded in paraffin, cut into 7- $\mu$ m sections, and stained with hematoxylin and eosin for histology. Fiber area and number were calculated by averaging the counts of five separate fields in four distinct areas in each specimen by a blinded observer using ImageJ software (National Institutes of Health, Bethesda, Md).

**Capillary density analysis.** Tissue sections were deparaffinized and stained using a primary antibody against CD31, followed by application of a fluorophore-conjugated second-



**Fig 2.** Mononuclear cell (MNC) treatment ( $5 \times 10^5$ ) improves hind limb ischemia (MNC-treated,  $n = 21$ ; control,  $n = 20$ ). **A**, MNC-treated mice showed improved functional outcomes according to Tarlov score ( $*P = .0004$ ). **B**, MNC-treated mice had greater improvement of ischemia as shown by ischemia score ( $P = .0002$ ). **C**, No significant differences were observed for the modified ischemia score ( $P = .4587$ ). Invasive Doppler measurements show MNC-treated mice had improved (**D**) proximal flow ( $*P < .0001$ ) and (**E**) distal flow ( $*P < .0001$ ). **F**, Representative laser Doppler images show improved perfusion in MNC-treated mice. The arrows show improved perfusion in MNC-treated mice at days 7 and 14. *P* values represent statistical analysis by analysis of variance. The error bars show the standard error of the mean.

ary antibody. An antifade mounting medium containing 4',6-diamidino-2-phenylindole was applied before cover slips were placed. Capillary density, defined as number of capillaries per muscle fiber, was counted manually by a blinded observer using ImageJ software in five different randomized fields per slide (original magnification,  $\times 40$ ).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, Calif). Results are expressed as the mean  $\pm$  standard error of the mean. Comparisons between groups were performed using analysis of variance, with post hoc testing performed with Bonferroni analysis or unpaired *t*-tests, as appropriate. Values of  $P < .05$  were considered statistically significant.

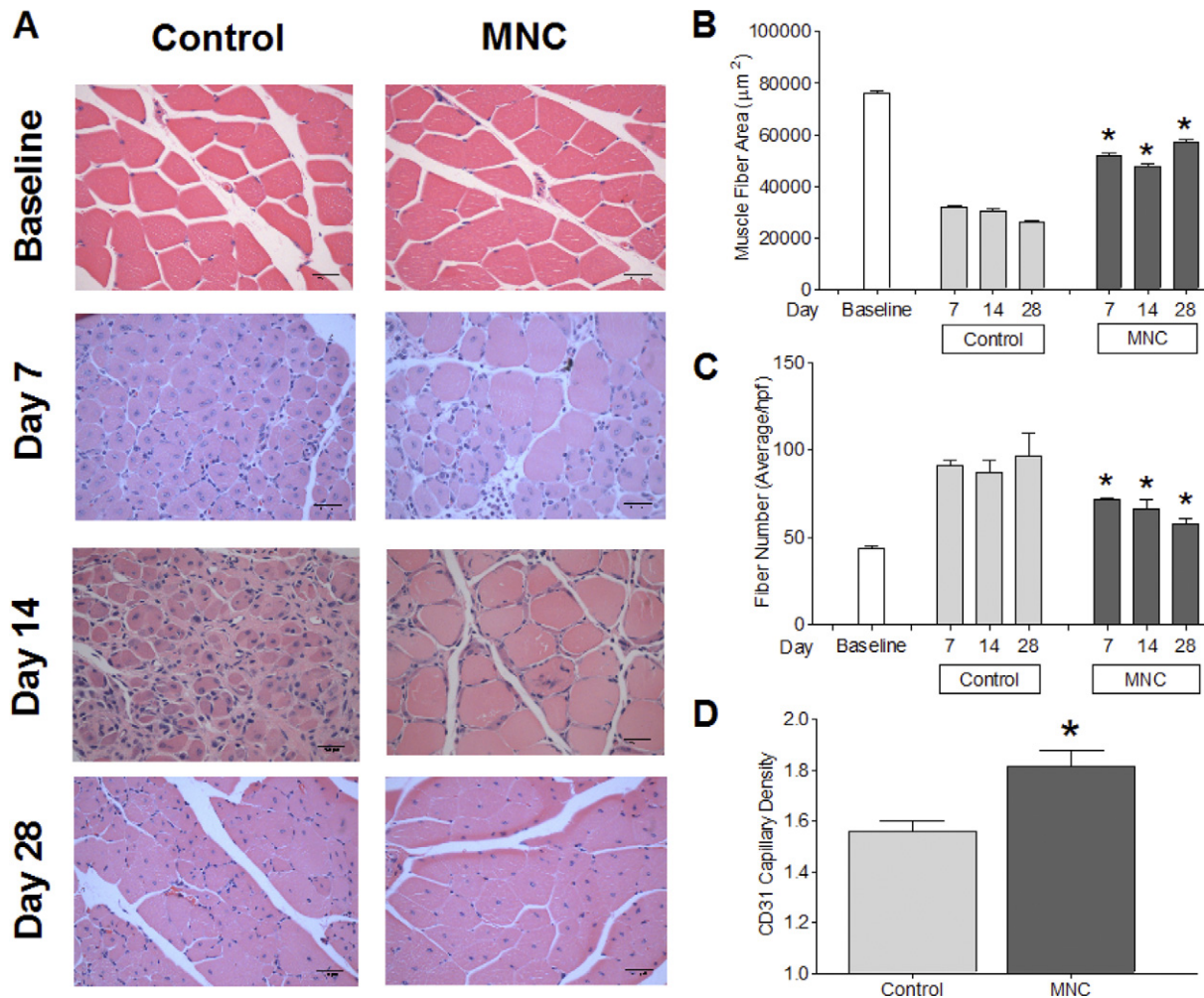
## RESULTS

**MNCs and EPCs are present in bone marrow isolates.** MNCs were isolated using a protocol similar to one used in clinical practice.<sup>33</sup> Flow cytometry showed that

$90.74\% \pm 1.67\%$  of the murine bone marrow cells isolated with this protocol were CD45<sup>+</sup> leukocytes. Further characterization of this isolate found it to be CD115<sup>+</sup> and CD11b<sup>+</sup> ( $46.65\% \pm 6.41\%$ ; Fig 1, C and D). EPC markers were found at varying amounts, with high levels of expression of VEGFR2 ( $75.55\% \pm 6.10\%$ ) but lower levels of CD34 ( $14.29\% \pm 1.32\%$ ) and CD133 ( $7.48\% \pm 3.10\%$ ; Fig 1, E and F); cells were most frequently dual-positive for CD45 and VEGFR2 (Fig 1, G and H). These results suggest that MNCs derived from a clinically used protocol have a large number of monocytes with varying quantities of EPCs.

**MNC treatment improves outcomes in the murine hind limb ischemia model.** Hind limb ischemia was induced acutely in mice using common femoral artery ligation and superficial femoral artery excision (Fig 1, A), producing reproducible toe gangrene (Fig 1, B). Mice were treated immediately afterward with control vehicle without





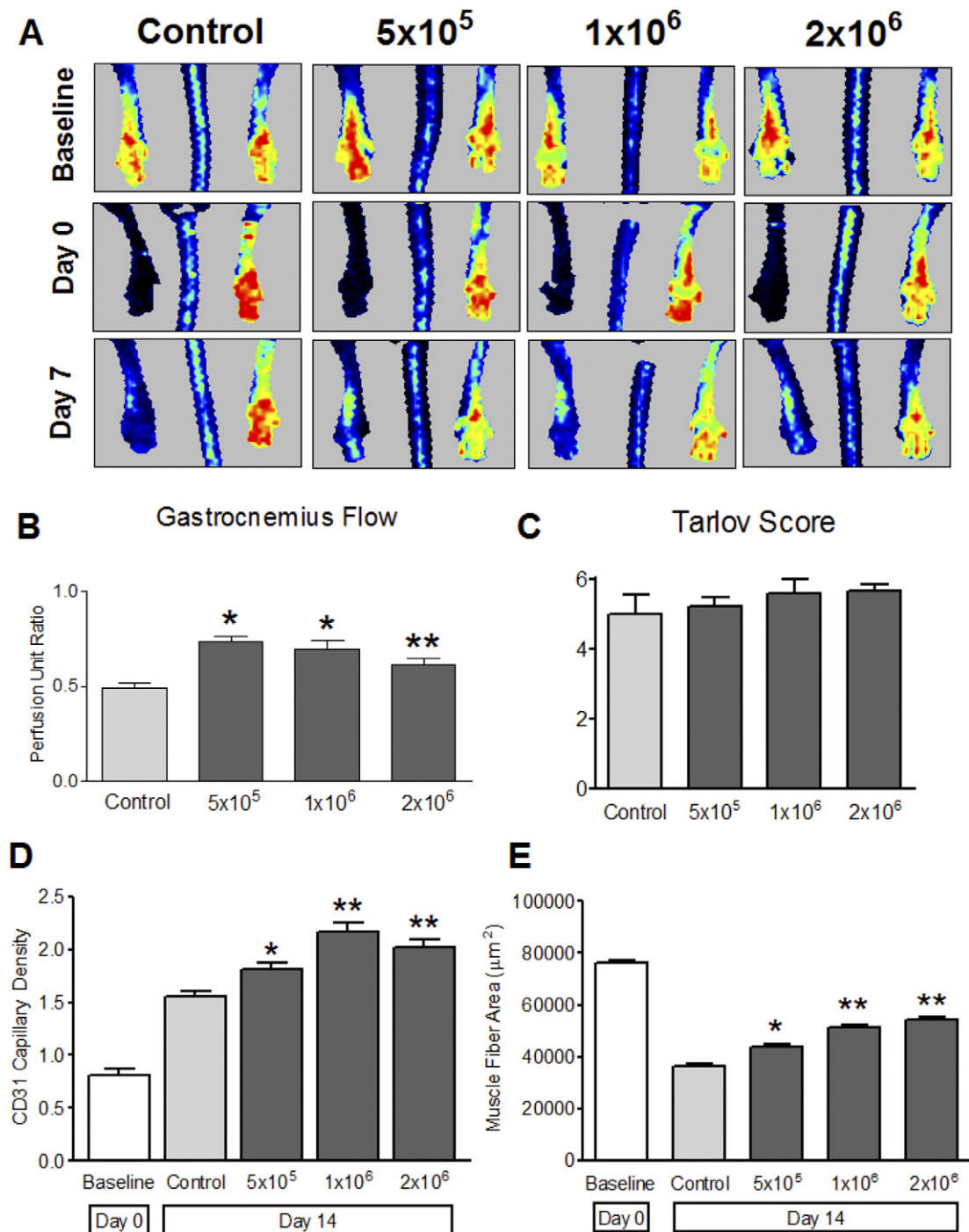
**Fig 3.** Gastrocnemius muscle histology in mice treated with mononuclear cells (MNCs;  $n = 3$ ) and in controls ( $n = 3$ ). **A**, Histologic comparison of control (left) and MNC-treated (right) mice over 28 days (stained with hematoxylin and eosin; original magnification,  $\times 40$ ). **B**, MNC-treated mice demonstrated increased muscle fiber area at days 7, 14, and 28 ( $*P = .0053$ ). **C**, MNC-treated mice showed decreased number of muscle fibers ( $*P < .0001$ ). **D**, MNC-treated mice demonstrated higher capillary density at day 14 ( $*P < .0001$ ). *P* values represent statistical analysis by analysis of variance. The error bars show the standard error of the mean.

any MNCs or with  $5 \times 10^5$  MNCs injected into the gastrocnemius muscle. Mice treated with MNCs and control mice showed equally diminished proximal and distal flow on day 0 after femoral artery ligation compared with the contralateral leg (proximal:  $0.39 \pm 0.02$  vs  $0.40 \pm 0.03$  perfusion units [ratio ischemic:control],  $P = .8305$ ; distal:  $0.28 \pm 0.01$  vs  $0.27 \pm 0.01$  perfusion units [ratio ischemic:control],  $P = .6904$ , *t*-test).

Over 28 days, MNC-treated mice showed improved functional outcomes compared with control mice, with accelerated improvement in the Tarlov score at day 7 ( $5.43 \pm 0.21$  vs  $4.37 \pm 0.23$ ,  $P = .0017$ , *t*-test; Fig 2, A). The grade of ischemia improved in MNC-treated mice using the ischemia score ( $P = .0002$ ) but not the modified ischemia score ( $P = .456$ ; Fig 2, B and C). Mice treated with MNCs showed significantly augmented proximal and distal per-

sion, as measured at day 7 with invasive Doppler (proximal semimembranosus flow:  $0.87 \pm 0.04$  vs  $0.73 \pm 0.05$ ,  $P = .0429$ ; distal gastrocnemius flow:  $0.61 \pm 0.03$  vs  $0.51 \pm 0.02$ ,  $P = .0024$ , *t*-test; Fig 2, D and E) or with noninvasive laser Doppler (Fig 2, F).

Histologic findings correlated with vascular and functional outcomes (Fig 3, A). MNC-treated mice showed increased gastrocnemius muscle fiber area compared with control mice ( $P = .0053$ ; Fig 3, B). In addition, MNC-treated mice showed fewer muscle fibers per high-power field compared with control mice ( $P < .0001$ ; Fig 3, C). Angiogenesis was measured in histologic sections by direct assessment of capillary density; at day 14, MNC-treated mice showed increased gastrocnemius capillary density compared with control mice ( $P = .0004$ , *t*-test; Fig 3, D). These data show increased



**Fig 4.** Mononuclear cell (MNC) dose-response ( $n = 4$  to  $6$  per group). **A**, Quantification of representative laser Doppler perfusion images showed no differences between MNC-treated groups at day 7 ( $P = .7501$ , analysis of variance [ANOVA]). **B**, MNC-treated mice show increased perfusion (day 7) compared with control mice ( $P = .0018$ , ANOVA);  $5 \times 10^5$  and  $1 \times 10^6$  MNC groups showed increased distal flow vs control ( $*P < .05$ , post hoc test;  $**P > .05$  vs other groups). **C**, No significant differences between MNC-treatment groups and control were seen in functional outcomes ( $P = .5520$ , ANOVA). **D**, Capillary density was increased at day 14 in MNC-treated mice vs control mice ( $n = 3$ ;  $P < .0001$ , ANOVA;  $*P < .05$  vs control, post hoc test;  $**P < .05$  vs control, post hoc test;  $P > .05$  vs each other, post hoc test). **E**, Gastrocnemius muscle fiber area was increased at day 14 in MNC-treated mice vs control mice ( $P < .0001$ , ANOVA;  $*P < .05$  vs control, post hoc test;  $**P < .05$  vs control, post hoc test;  $P > .05$  vs each other, post hoc test). The *error bars* show the standard error of the mean.

angiogenesis in MNC-treated mice compared with control mice, consistent with the data showing increased limb perfusion (Figs 2, B, D, E, and F).

**Increased MNC dose affects outcomes.** To demonstrate the relevance of the animal model, we evaluated a question of clinical significance: Does the number of MNCs injected make a difference in outcome? Accordingly, dose-response groups were evaluated. MNC-treated mice received gastrocnemius muscle injections with doses of  $5 \times 10^5$ ,  $1 \times 10^6$ , or  $2 \times 10^6$  MNCs, with control mice receiving no cells. Laser Doppler perfusion imaging of the distal foot on day 7 showed increased perfusion in the MNC-treated mice compared with control mice, but without any significant differences between the treatment groups ( $P = .7501$ ; Fig 4, A). Quantification, as assessed by invasive Doppler flow imaging, showed all MNC treatment groups ( $5 \times 10^5$ ,  $1 \times 10^6$ , and  $2 \times 10^6$ ) displayed increased distal flow compared with control mice ( $P = .0018$ ; Fig 4, B), similar to the findings with noninvasive laser Doppler (Fig 4, A). The groups showed no differences in functional scores (Tarlov:  $P = .5520$ ; ischemia score:  $P = .4444$ ; Fig 4, C).

Despite the lack of gross functional responses with higher MNC doses, there was evidence of response on the microscopic level (Fig 4, D and E). At day 14, the MNC-treated mice demonstrated increased capillary density compared with control mice ( $P < .0001$ ; Fig 4, D). The highest capillary density was observed in the  $1 \times 10^6$  MNC-treatment group, with no significant difference in capillary density observed between the  $1 \times 10^6$  and  $2 \times 10^6$  MNC-treatment groups ( $P > .05$ ; Fig 4, D).

Histologic evaluation of gastrocnemius muscle fiber area suggested a correlation between improved gastrocnemius muscle architecture and treatment with increased MNC concentrations. At day 14, all MNC-treated mice showed increased gastrocnemius fiber area compared with control mice ( $P < .0001$ ; Fig 4, E). Gastrocnemius fiber area was statistically increased in the  $1 \times 10^6$  and  $2 \times 10^6$  MNC-treatment groups compared with the  $5 \times 10^5$  MNC-treatment group and control mice ( $P < .05$ ) but without difference between the  $1 \times 10^6$  and  $2 \times 10^6$  MNC-treatment groups ( $P > .05$ ). Similar outcomes were seen between the MNC-treated mice and control mice in gastrocnemius muscle fiber number, and groups treated with higher doses of MNCs showed diminished fiber numbers compared with control mice ( $P = .0002$ ). These results suggest that increased dose of injected MNC have effects on the microcirculation but may be inadequate at the doses examined to affect functional scores or gross perfusion.

**Proximal MNC injections influence distal ischemia outcomes.** We also examined another clinical question: Does a more proximal injection site, proximal to the ischemic area, affect the response? Treatment groups received  $5 \times 10^5$  MNC cells that were injected proximally into the semimembranosus muscle (Fig 1, A), whereas control mice received no cells. No statistically significant differences were observed in the functional or ischemic scores between the proximally injected and distally injected mice (Fig 5, A and B).

At day 7, flow in the proximal muscles was higher in the MNC-treated mice than in control mice ( $P = .0041$ ), with no difference between proximal and distal injection ( $P > .05$ ; Fig 5, C). However, flow in the distal muscles was increased in both proximally injected and distally injected mice compared with control mice ( $P = .0086$ ), without a difference between proximal and distal injections ( $P > .05$ ; Fig 5, D).

Similar to these data, the proximally injected MNC-treated mice showed higher capillary density at day 14 than control mice ( $P < .0001$ ) but without any significant differences in capillary density compared with the distally injected group ( $P > .05$ ; Fig 5, E).

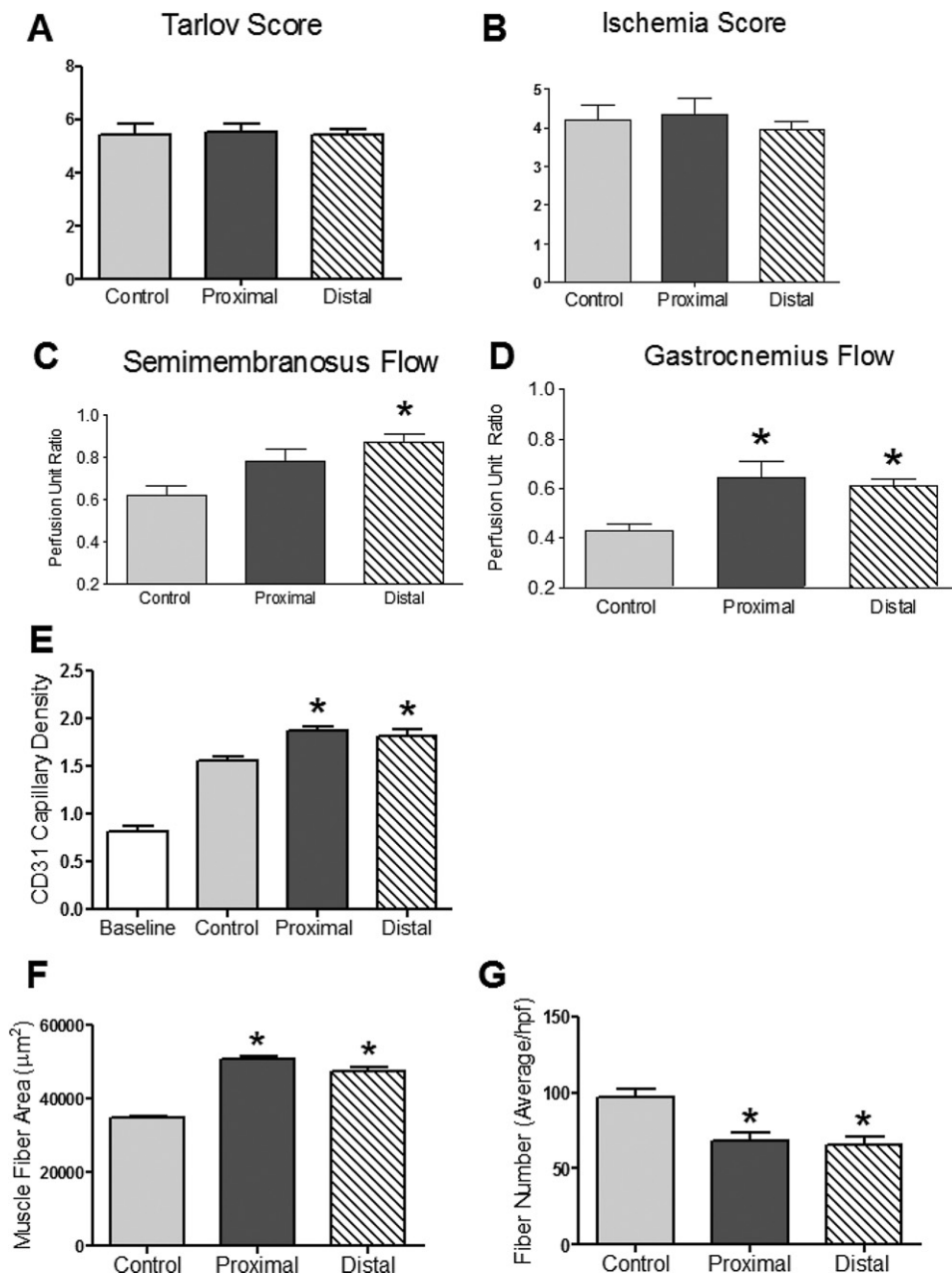
Histologic evaluation of the gastrocnemius muscle at day 14 demonstrated increased fiber area in MNC-treated mice than in control mice ( $P < .0001$ ) and in proximally injected mice compared with distally injected mice ( $P < .05$ ; Fig 5, F). Similarly, gastrocnemius fiber number was decreased at day 14 in MNC-treated mice compared with control mice ( $P = .002$ ), without any significant differences in fiber number between proximally injected and distally injected mice ( $P > .05$ ; Fig 5, G). These data are consistent with stem cell injections improving the kinetics of recovery from hind limb ischemia, despite the limitations of our mouse model.

## DISCUSSION

New clinical studies suggest that stem cell injection for CLI is a valid therapeutic option in selected patients. Despite supporting clinical evidence, conflicting clinical protocols and ignorance regarding stem cell biology have tempered adoption of stem cell therapy in clinical practice. We describe an easy-to-perform preclinical model to test different parameters of stem cell therapy. We show that MNC treatment provided a more rapid improvement in perfusion in this ischemic model, that a dose-response curve is likely to exist for stem cells, and that the site of stem cell injection may have functional consequences.

The rationale for this study was to establish an acute murine model of severe ischemia that could accommodate the testing of several clinical parameters important to clinical trials of cell therapy. A study demonstrating six different ischemia models concluded that simple ligation of the femoral artery at the level below the deep femoral (profunda) artery is most suitable for studying chronic mild ischemia.<sup>27</sup> This model resulted in a range from no necrosis to toe necrosis. Many articles in the literature use the severe ischemia model, which consists of femoral artery ligation with excision of all side branches.<sup>27,34-40</sup> This model results in severe ischemia with profound necrosis, ranging from toe necrosis to autoamputation of limbs. Stripping the femoral artery from the distal site of bifurcation of the deep femoral artery to the saphenous artery is most suitable for a model of severe ischemia.<sup>27</sup> The deep arterial system remains intact, however, and blood flow is redirected through this route.

We believe our model more closely resembles diseases in humans where arteries are occluded but still present. This can result in arteriogenesis as well as angiogenesis. We adopted a variation of the arterial stripping model as well as interrupting collateral flow through the deep system by



**Fig 5.** Comparison of response to proximal vs distal mononuclear cell (MNC) injection ( $n = 21$ ) vs control ( $n = 6$ ). **A**, Functional measurements with the Tarlov scale (day 7) showed no difference between control and MNC-treated groups ( $P = .9821$ ). **B**, Functional measurements with the ischemia scale (day 7) showed no difference between groups ( $P = .6594$ ). **C**, Increased semimembranosus flow with MNC-treated mice ( $P = .0041$ , analysis of variance [ANOVA]); there was no significant difference in flow between proximal and distal treatment ( $P > .05$ , post hoc test;  $*P < .05$  vs control, post hoc test). **D**, Distal flow was increased in proximally and distally treated mice vs control ( $P = .0086$ , ANOVA). There was no significant difference in flow between proximal and distal MNC treatment ( $P > .05$ , post hoc test;  $*P < .05$  vs control, post hoc test,  $n = 3$ ). **E**, MNC-treated mice showed increased capillary density compared with control and baseline ( $P < .0001$ , ANOVA); there was no significant difference in capillary density between proximally and distally treated mice ( $P > .05$ , post hoc test;  $*P < .05$  vs control, post hoc test,  $n = 3$ ). **F**, MNC-treated mice showed increased gastrocnemius muscle fiber area compared with control mice ( $P < .0001$ , ANOVA). The difference between proximal and distal injection was significant ( $P < .05$ , post hoc test;  $*P < .05$  vs control, post hoc test,  $n = 3$ ). **G**, MNC-treated mice showed decreased gastrocnemius muscle fiber number compared with control mice ( $P = .002$ , ANOVA); the difference between proximal and distal injection was not significant ( $P > .05$ , post hoc test;  $*P < .05$  vs control, post hoc test,  $n = 3$ ).



**Table.** Functional scoring

Score	Description
Tarlov score	
0	No movement
1	Barely perceptible movement, non-weight-bearing
2	Frequent movement, non-weight-bearing
3	Supports weight, partial weight-bearing
4	Walks with mild deficit
5	Normal but slow walking
6	Full and fast walking
Ischemia score	
0	Autoamputation >half lower limb
1	Gangrenous tissue >half foot
2	Gangrenous tissue <half foot, with lower limb muscle necrosis
3	Gangrenous tissue <half foot, without lower limb muscle necrosis
4	Pale foot or gait abnormalities
5	Normal
Modified ischemia score	
0	Autoamputation of leg
1	Leg necrosis
2	Foot necrosis
3	Discoloration of >2 toes
4	Discoloration of 1 toe
5	Discoloration of >2 nails
6	Discoloration of 1 nail
7	No necrosis

ligation above the branches. Through the use of this modified model, we were able to consistently achieve distal hind limb tissue necrosis and thus more accurately represent chronic manifestations of atherosclerotic disease (Fig 1, B). However, small technical variations in this ischemia model have physiologic consequences.<sup>29</sup> With excision of all branches, arteriogenesis cannot be studied because all pre-existing connections of arterioles to the vascular tree are disrupted and cannot be repaired.

The lack of an in-depth understanding regarding EPCs and their lineage continues to be problematic. Clinical observations that cell therapies based on whole bone marrow appear to be more successful than isolated cell subpopulations<sup>6,24</sup> have led to the idea that several different bone marrow cell types, all sharing a common monocytic phenotype, are necessary for successful angiogenesis. Clinical studies have traditionally relied on the use of density gradient centrifugation to isolate MNC fractions from whole bone marrow.<sup>41</sup> Therefore, we used this method to isolate the murine MNCs to be tested in this study. Human studies have documented the composition of the clinically used bone marrow-derived MNC isolate and have found a heterogeneous population of cell types that includes hematopoietic stem cells (CD34<sup>+</sup>), MNCs (CD14<sup>+</sup>, CD11b, CD115), and EPCs (CD133<sup>+</sup>, VEGFR2<sup>+</sup>).<sup>41,42</sup> Using C57BL/6 bone marrow, we were able to closely replicate clinical cell therapy marrow isolates. Analysis of our marrow

isolate found it was primarily mononuclear in composition (CD45<sup>+</sup>, CD11b<sup>+</sup>), with elimination of granulocytes achieved through gradient centrifugation. Similar to clinical studies, we found varying numbers of EPCs (CD34<sup>+</sup>, CD133<sup>+</sup>, VEGFR2<sup>+</sup>) as well as colocalization of the leukocyte-common antigen CD45<sup>+</sup> with progenitor cell markers (CD34<sup>+</sup>, CD133<sup>+</sup>, VEGFR2<sup>+</sup>). The ability to closely replicate human cell therapy using C57BL/6 bone marrow enhances the clinical utility of this mouse model.

Our results are similar to those reported in several clinical trials.<sup>7,8,10,11,43</sup> We observed improvements in function and in limb perfusion with MNC treatment (Fig 2), as well as augmented muscle fiber regeneration with evidence of angiogenesis (Fig 3). In previous reports, the number of injected MNCs used to treat CLI has been variable, and interestingly, positive effects on perfusion have been observed even when low cell numbers were used.<sup>6</sup> Only one study to date has attempted to establish an association between clinical response and cell number; the authors observed a correlation between the number of CD34<sup>+</sup> cells injected and improvements in ankle-brachial index and concluded that the number of injected CD34<sup>+</sup> cells was a primary factor influencing the clinical efficacy of cell therapy.<sup>14</sup> Our findings show a similar correlation, with the  $5 \times 10^5$  MNC-treatment group showing improved flow compared with control mice and the higher  $1 \times 10^6$  MNC-treatment group showing improved flow over the control group and the  $5 \times 10^5$  MNC-treatment group (Fig 4). This positive correlation, however, appears to plateau at a certain cell number, perhaps secondary to a heightened inflammatory response that interferes with the angiogenic potential of the EPCs, although the exact mechanism is unclear.

Clinical trials using intramuscular and intravascular injections, or a combination of both, have produced favorable results; however, intramuscular injection into the gastrocnemius muscle has been the preferred application in most trials.<sup>7,44-46</sup> In our study, more proximally injected MNCs showed similar increase in muscle flow and capillary density compared with the more distal injection sites (Fig 5). Interestingly, proximal injection did result in increased significantly improved muscle area compared with control and distal injection (Fig 5, F); however, there was no overall increase in limb ischemia or function (Fig 5, A and B). We therefore believe that more proximal injection of MNCs has the potential for greater therapeutic effect and that the numbers of MNCs likely need to be increased to achieve greater functional outcomes. This may be tempered by the plateau in effect with increased doses of cells (Fig 4), suggesting that cell dose and injection location need to be optimized simultaneously.

Although we believe that our model of murine hind limb ischemia has clinical value, we likewise recognize its limitations. Despite the added modification of total SFA excision, this model continues to be acute in nature. In addition, without additional treatment, C57BL/6 mice spontaneously regain near-maximal flow within several weeks after ligation. This quick recovery from ischemia makes it more difficult to quantify and observe subtle flow

and functional recovery as a result of cell therapy; it is precisely this inherent ability for spontaneous angiogenesis that may mask improvements in some of our treatment groups. Other improvements that could be made to our model include use of animal models of disease, such as apolipoprotein E-knockout mice, use of aged animals to mimic aged human patients, and use of other mouse strains that have different features of human disease.<sup>31</sup>

The mouse demonstrates spontaneous recovery from hind limb ischemia, limiting the ability to discriminate between changes in the final end point and, thus, the ability to measure gross changes in tester parameters. However, we believe our model shows that cellular therapy provides a kinetic advantage, with faster recovery from ischemia with parameters measured between 7 and 14 days. Finally, *ex vivo* treatment of isolated MNCs with cell culture conditions or drugs, such as statins or growth factors, may mimic other clinical protocols used in some trials.

## CONCLUSIONS

Our study shows that high femoral ligation with complete excision of the SFA is a reproducible model of hind limb ischemia in C57BL/6 mice. Similar to human trials, injection of MNCs into murine ischemic limbs improves vascular and functional outcomes. Our data suggest that cell number, type, and location of injection can be optimized. As such, it is likely that preclinical animal models will continue to serve an important role in comparing factors of clinical utility.

## AUTHOR CONTRIBUTIONS

Conception and design: RB, MB, PH, CP, XL, WL, MC, AD

Analysis and interpretation: RB, CJ, MB, CP, XL, WL, MC, AD

Data collection: RB, CJ, MB, PH, CP, MC

Writing the article: RB, CJ, MB, PH, XL, WL, AD

Critical revision of the article: RB, CJ, CP, MC, AD

Final approval of the article: RB, CJ, MB, PH, CP, XL, WL, MC, AD

Statistical analysis: RB, CJ, MB, AD

Obtained funding: AD

Overall responsibility: AD

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## DISCUSSION

**Dr Darwin Eton** (*Chicago, Ill*): How do you know the cells are still there 24 hours or 48 hours after injection? Is this a cell therapy, or is it actually a drug delivery system where the cells get into the tissue, lyse, and release all the cytokines into the tissue? How many mice were there in each group?

**Dr Robert A. Brenes**. Dr Eton, thank you for your questions. In our study, we did not evaluate if the cells are present 24 to 48 hours after injection; therefore, I am unable to comment on this. We also did not evaluate if it is the effector cells or the cytokines released into the tissue that lead to angiogenesis. However, we believe it may be a combination of the mononuclear cells, endothelial progenitor cells, as well as the cytokines that all contribute to the angiogenesis seen. In our primary experiment, we had an n value of 20 mice, with a few mice removed at each time point for histological analysis.

**Dr Dai Yamanouchi** (*Madison, Wisc*). I have one question about the mouse model. You showed in the limitation slide that

this is the acute limb ischemia, not the chronic limb ischemia. However, the chronic limb ischemia model can be made and actually has been utilized by waiting for 4 weeks or 6 after the initial surgery before starting the treatment. Is this model as durable to wait like 4 or 6 weeks to create chronic limb ischemia?

**Dr Brenes**. Dr Yamanouchi, thank you for your comments. We recognize that our model, as well as others described in the literature, are acute models of limb ischemia. C57BL/6 mice regain their flow spontaneously without cell injection by 4 to 6 weeks post ligation. Therefore, one must study the treatment modality early after inducing ischemia to see maximal effect. Interestingly, our lab has shown that aged mice have a delayed spontaneous recovery to flow as well as functional score. We believe these mice may be a better model to test cell intervention in more of a "chronic" setting with delayed injections.